

(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
3 June 2004 (03.06.2004)

PCT

(10) International Publication Number  
**WO 2004/046367 A1**

- (51) International Patent Classification<sup>7</sup>: **C12P 3/00**, (74) Agent: VAN WESTENBRUGGE, Andries; Nederlandsch Octrooibureau, Scheveningsweg 82, P.O. Box 29720, NL-2502 LS The Hague (NL).  
C02F 3/34
- (21) International Application Number:  
PCT/NL2003/000818
- (22) International Filing Date:  
20 November 2003 (20.11.2003)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
02079845.0 20 November 2002 (20.11.2002) EP
- (71) Applicants (for all designated States except US): **WAGENINGEN UNIVERSITY** [NL/NL]; P.O. Box 9101, NL-6700 HB Wageningen (NL). **PAQUES B.V.** [NL/NL]; P.O. Box 52, NL-8560 AB Balk (NL).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **STAMS, A., J., M.** [NL/NL]; Gerdessastraat 8, NL-6701 AK Wageningen (NL). **BUISMAN, Cees, Jan, Nico** [NL/NL]; Stinsenwei 1, NL-8571 RH Harich (NL). **DIJKMAN, Henk** [NL/NL]; Geeuwkade 8, NL-8651 AA Ijlst (NL). **DE VOS, Willem, M.** [NL/NL]; Langhoven 50, NL-6721 SJ Bennekom (NL).
- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (regional): ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:  
— with international search report  
— with amended claims
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PROCESS FOR ANAEROBIC OXIDATION OF METHANE

(57) Abstract: Methane can be used as a source for producing hydrogen or hydrogen equivalents in a biological process using mesophilic or thermophilic bacteria of the order of the *Thermotogales*, e.g. *Thermotoga maritima*. Hydrogen can be produced as such, or as a reducing equivalent for the reduction of various compounds such as sulphur compounds. Especially, the methane oxidation can be used for anaerobically reducing sulphate to sulphide using methane as the predominant or sole electron donor, in coculture with sulphate-reducing micro-organisms.

WO 2004/046367 A1

## Process for Anaerobic Oxidation of Methane

### *Field of the invention*

The present invention relates to an oxygen-free biological process for converting methane to hydrogen or hydrogen equivalents. Furthermore, the invention relates to a biological process of reducing sulphur compounds to sulphide.

### *Background*

Biological methane oxidation in the presence of oxygen is a well-established process in natural habitats and in industrial applications. It is reported that aggregates of archaea and sulphate-reducing bacteria are capable of methane oxidation in deep-sea conditions (Boetius et al, *Nature*, 407: 623-626 (2000), Hoehler et al, *Global Biogeochemical Cycles*, 8: 451-563 (1994). However, no pure or defined microbial cultures are known that are capable of anaerobic methane oxidation (Orphan et al., *Proc. Natl. Acad. Sci. USA* (2002), 99, 7663-7668). Hence, anaerobic oxidation of methane is not well-understood and is not applied on an industrial scale. The use of archaea in an industrial process is hardly feasible, if at all, because of their extremely low growth rates. (?)

Removal of sulphur oxide compounds such as sulphate, sulphite, sulphur dioxide, thiosulphate and the like and elemental sulphur by anaerobic conversion to sulphide at moderate or high temperature is well known, e.g. from EP-A-0451922, WO 92/17410, WO 93/24416 and WO 98/02524. These processes usually require an electron donor (or hydrogen donor) which can be hydrogen, carbon monoxide or organic molecules such as alcohols and fatty acids.

It was found recently (Balk et al., *Int. J. Syst. Evol. Microbiol.* (2002), 52, 1361-1368) that certain *Thermotoga* species are capable of anaerobically degrading methanol, alone, in coculture with *Methanothermobacter* or *Thermodesulfovibrio* species, or in the presence of sulphur, sulphur oxide compounds or organic sulphur compounds, such as anthraquinone-2,6-disulphonate.

### *Summary of the invention*

It was found according to the invention that anaerobic thermophilic bacteria of the order of the *Thermotogales*, especially from the species *Thermotoga*, are capable of converting methane, in the absence of oxygen, to hydrogen or hydrogen equivalents. The methane carbon atom was found to be converted to carbon dioxide and was not incorporated in the biomass. The hydrogen produced can be used as such, or can be

used to provide hydrogen equivalents suitable for reducing various compounds e.g. sulphur compounds such as sulphate, sulphite and thiosulphate. It was furthermore found that hydrogen equivalents required for biological reduction reactions can be effectively provided by methane oxidation by anaerobic methane-oxidising bacteria.

5 Thus the invention concerns a process of producing hydrogen or hydrogen equivalents by anaerobically subjecting methane to the activity of one or more *Thermotogales* species. Similarly, the invention concerns a process of anaerobic oxidising methane using a *Thermotogales* species or strain. Furthermore, the invention concerns a process for biological reduction of chemicals such as sulphur compounds and metals, wherein

10 the required hydrogen equivalents are produced by subjecting methane to anaerobic methane-oxidising bacteria.

#### *Description of the invention*

The invention pertains to the anaerobic, bacterial production of hydrogen or hydrogen equivalents. In the present context, hydrogen equivalents are understood to

15 comprise atoms, molecules or electrons, i.e. reduction equivalents, which lower the oxidation state of a substrate. These include e.g. acetate and formate. They are also referred to as electron donors. Where the present process produces hydrogen equivalents, as distinct from (molecular) hydrogen, the process is carried out in the presence of a suitable substrate capable of accepting the hydrogen equivalents. Where

20 reference is made to methane, also higher alkanes and alkenes, such as ethane, ethene, propane, etc. are contemplated.

The bacteria to be used according to the invention are anaerobic methane-oxidising (alkane-oxidising) bacteria. These bacteria include terrestrial and aquatic (marine) species, which can be obtained from hydrothermal sources, oil-wells, and

25 sometimes anaerobic thermophilic bioreactors. They can grow under a variety of environmental conditions and, depending on the natural source and possibly adaptation processes, they can be mesophilic and/or thermophilic. Examples of suitable bacteria belong to the order of the *Thermotogales*, which are mostly thermophilic. A description thereof is given by Wery et al. (*FEMS Microbiol. Biol.* 41, (2002) 105-114) and

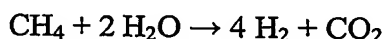
30 Reysenbach et al. (*Int. J. Syst. Evol. Microbiol.*, 52, (2002) 685-690). Their use in producing hydrogen from organic sources such as sugars is described in WO 02/06503. The *Thermotogales* include the genera *Marinitoga*, *Geotoga*, *Petrotoga*, *Thermotoga*, *Thermosipho* and *Fervidobacterium*. They are especially from the group containing the

latter three genera. These include the species (with DSM accession numbers) *Thermotoga maritima* (DSM 3109), *Thermotoga thermarum* (DSM 5069), *Thermotoga hypogea* (DSM 11164), *Thermotoga subterranea* (DSM 9912), *Thermotoga elfei* (DSM 9442), *Thermotoga lettingae* (DSM 14385), *Thermosipho melanesiensis* (DSM 12029),  
5 *Thermosipho geolei* (DSM 13256), *Fervidobacterium islandicum* (DSM 5733) and *F. nodosum* (DSM 5306). Most of them are available in recognised culture collection such as DSM or ATCC, and the genome of some of them, such as *Thermotoga maritima*, has been sequenced (Nelson et al., *Nature* (1999), 399, 323-329).

The methane-oxidising bacteria may be used as a pure culture of one of the  
10 species or strains mentioned above or as a defined mixture with other bacteria, or as a part of a mixed culture obtained from environmental samples or from bioreactors, if necessary and preferably after adaptation to the desired process conditions. The use of a pure culture has the advantage of allowing the process to be controlled as desired. The invention also concerns such pure cultures as well as defined combinations of cultures  
15 as further illustrated below.

The species to be used in the process of the invention are mesophilic or thermophilic species. The thermophilic species have their maximum activity between 50 and 100°C, but they are generally sufficiently active in the mesophilic temperature range for the process to be carried out at temperatures between 30 and 50°C as well, or even  
20 from 25°C upwards, if necessary after adaptation. Mesophilic species have their maximum activity between 30 and 50°C, but are sufficiently active from 20°C and up to e.g. 60°C. The most preferred temperatures for the process of the invention are between 25 and 90°C, most preferably between 30 and 60°C.

In an embodiment of the process of the invention, the anaerobic methane  
25 oxidation is performed for producing molecular hydrogen. The relevant total reaction can be simplified as follows:



The culture medium contains basic mineral medium supplemented with growth factors into which methane is introduced e.g. by sparging or another method that ensures  
30 intimate contact with the micro-organisms. The hydrogen produced can be collected e.g. using gas recirculation, wherein the gas is contacted with a selective membrane which is permeable for hydrogen and impermeable for larger molecules including methane, and the remaining gas can be recirculated to the methane-oxidising reactor. Alternatively,

suitable selective absorbents can be arranged in such a manner that the gas evolving from the reactor is contacted with the absorbents. Efficient withdrawal of hydrogen from the reaction mixture ensures sufficient bioconversion of methane to hydrogen. The hydrogen produced can be used as a fuel or as a chemical synthesis agent or in biological or chemical reduction processes.

In a preferred embodiment, the anaerobic methane oxidation is performed for reducing substrates, such as nitrate, azo compounds, inorganic and organic sulphur compounds such as elemental sulphur, sulphate, sulphite, thiosulphate, polysulphides, anthraquinone-2,6-disulphonate, dissolved metals, oxidised halogen compounds, nitrate and other compounds that must be removed. The compounds can be present in liquid waste streams, if appropriate after extraction from the gas stream by scrubbing or the like. They can also be present e.g. as soil contaminants. The compounds to be reduced can also be present in production lines, for producing desired reduced compounds. The following reaction may apply:



wherein A is a hydrogen acceptor, and  $\text{AH}_2$  may be replaced by equivalents or subsequent conversion products. According to this embodiment, methane-oxidising bacteria to be used include those of the *Thermotogales* order as described above, as well as other methane-oxidisers, e.g. those related to *Desulfosarcina*. The reduction step itself is in particular a biological reduction using suitable respiring organisms. This process is schematically illustrated in figure 1.

In a particularly preferred embodiment, the anaerobic methane oxidation is used to reduce oxidised sulphur compounds, such as sulphate, sulphite and elemental sulphur. In the following, reference is made to sulphate, but other sulphur-oxygen species, such as sulphite and hydrogenated (e.g. bisulphite) and neutral (e.g. sulphur trioxide) equivalents are also comprised. The relevant total reaction can be simplified as follows:



This embodiment requires the presence of agents capable of transferring hydrogen equivalents to sulphate. Such agents are especially sulphate-reducing micro-organisms, which are known in the art. Suitable sulphate-reducing micro-organisms include mesophilic and thermophilic hydrogen-utilising strains from the bacterial sulphate-reducing genera, e.g. *Desulforomonas*, *Desulfovibrio*, *Thermodesulfovibrio* and *Desulfotomaculum* (e.g. the strain described in WO 98/02524) as well as the archaeal sulphate-reducing genus, e.g. *Archaeoglobus*, such as *A. profundus*.

The conversion of sulphate by a coculture comprising the anaerobic methane oxidisers as described above can be carried out in a conventional bioreactor having an inlet for sulphate-containing water, e.g. originating from a gas desulphurisation plant, a gas inlet for methane supply, a liquid outlet for sulphide-containing water, a gas outlet for the resulting gas mixture containing e.g. residual methane, hydrogen, hydrogen sulphide, and optionally means for supporting the biomass and for keeping it in effective contact with the liquid and (dissolved) gaseous materials, optional filters for separating gaseous products from the culture mixture and means for maintaining the desired reactor temperature. Furthermore, a gas separation unit may be provided for separating the resulting gas mixture and returning recovered methane as well as a treatment unit for treating hydrogen sulphide, e.g. a unit for biologically converting sulphide to elemental sulphur and for separating off the sulphur. Since the bacteria do not use methane for their cell synthesis, further carbon sources, e.g. methanol, ethanol, organic acids, yeast extract or components thereof, or other organic matter should be supplied to the bioreactor, in addition to methane.

Variations in the process of reducing sulphur compounds using a coculture of methane-oxidisers and sulphate-reducers are schematically illustrated in Figures 2-5. Fig. 2 is a flow diagram for reducing sulphate to sulphide, followed by biological oxidation of sulphide to elemental sulphur. Fig. 3 shows sulphate reduction in combination with metal precipitation in the form of metal sulphides (MeS) by the hydrogen sulphide produced and oxidation of the surplus hydrogen sulphide to elemental sulphur. Fig. 4 shows two variants of a process for producing hydrogen sulphide, either by separate stripping, or by hydrogen sulphide removal using the methane stream. The hydrogen sulphide can be concentrated and used for sulphuric acid production. Fig. 5 illustrates sulphur dioxide removal from gases by scrubbing (first stage) followed by biological reduction as in figure 2.

In another embodiment of the process of the invention can be used for reducing noxious bromate or chlorate to less noxious bromide and chloride. These compounds can be present in process water from chemical industries. The reduction of bromate or chlorate requires the presence of bromate- or chlorate-reducing species, which can be sulphate-reducing bacteria and archaea. Species capable of reducing chlorate or bromate those of the genera *Dechlorosoma*, *Dechloromonas* and *Pseudomonas* such as *Pseudomonas chloritidismutans*, *Dechloromonas agitata*, *Dechlorosoma suillum*, strain GR-1. Similarly, nitrate reduction can be performed using commonly known denitrifiers.

Known denitrifying bacteria include *Pseudomonas stutzeri*, *Paracoccus denitrificans*, *Haloarcula marismortui* and *Staphylococcus aureus*.

According to a further embodiment, the process may be used for reducing metal ions to their low-valence or zero-valence state. They can be precipitated and separated  
5 in these lower valence states e.g. as oxides, hydroxides, carbonates, phosphates, sulphides or neutral metals. The biological reduction of metals is described for example in WO 00/39035. Examples of metals that can be reduced and converted to insoluble metals or insoluble metal oxides, hydroxides or the like include selenium, tellurium, uranium, molybdenum, vanadium, chromium, and manganese. Bacteria capable of  
10 reducing these metals include the genera *Geobacter*, *Pseudomonas*, *Shewanella*, *Desulfovibrio*, *Desulfobacterium*, *Desulfomicrobium*, *Desulforomonas* and *Alteromonas*. If desired, a moving sand filter can be used for separating the resulting metal precipitates as described in WO 00/39035.

The use of the methane-oxidising bacteria in providing reducing equivalents in  
15 (biological) reduction processes is beneficial in technical and economical terms. Current process using methane as the ultimate reducing agent require the intermediary use hydrogen to be produced from methane by chemical (catalytic) reforming. This implies the investment in and use of reformers or similar equipment and also consumes about 50% of the methane by combustion needed to keep the catalytic process at the necessary  
20 high temperature. These drawbacks are eliminated by the present biological process, thus resulting in substantial cost savings both in equipment cost, and in operational cost (e.g. 50% lower methane consumption).

The process of the invention can be carried out in a conventional bioreactor of the anaerobic type, having means for introducing a gas into the reactor contents and  
25 means for carrying off gases from the headspace of the reactor. The reactor can be of the stirred type, but preferably the reactor is of a type having biofilms, present on carrier particles such as sand, basalt, polymer particles etc., or in the form of granules, plates, membranes and the like, in order to optimise contact between the substrate (methane) and the micro-organisms, and – in case of coculture – between the different micro-  
30 organisms.

An example of a suitable reactor type for the biological conversion is the so-called gaslift-loop reactor. This is a type of reactor which is especially beneficial when a gaseous substrate has to be supplied for the reaction. It is operated using a vertical circulation activated by the gas (methane) introduced at the bottom of the reactor.

An example of such a reactor is the 500 m<sup>3</sup> gaslift-loop reactor used at the zinc plant of Budel Zinc in the Netherlands. In this case 10 tons/day of sulphate is reduced biologically by addition of 12,000 nm<sup>3</sup>/day of hydrogen gas. Additionally, part of the bioreactor off-gas is recycled by compressors to improve the efficiency of the hydrogen utilisation.

5 Here daily 20000 nm<sup>3</sup> of natural gas is converted in a steam reforming unit in order to provide the required amount of hydrogen gas. When using anaerobic methane oxidation as described above, the reformer can be by-passed and methane can be introduced in the reactor directly.

Another suitable reactor type is a membrane bioreactor, wherein biomass

10 retention is effected by passing the reactor effluent through a (membrane) filter. A membrane bioreactor can also be useful for separating a gas product (such as hydrogen or hydrogen sulphide). In such an embodiment, a membrane which is permeable for the gas (e.g. hydrogen sulphide) separates reactor liquid from the gas space of the reactor, where an absorbing gas is passed for carrying away the gas. This allows low hydrogen

15 sulphide levels to be maintained in the reactor without requiring a high water flow through the reactor.

The process of the invention can be carried out at atmospheric pressure, or - if desired - at elevated pressure, e.g. pressure in the of 10-100 bar, using appropriate pressure-resistant equipment. Elevated pressures may have the advantage of increasing

20 the conversion rate of the biological processes using methane.

### Examples

**Strains.** *Thermotoga maritima* (DSM 3109) and *Archaeoglobus profundus* (DSM 5631) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, DE). *T. lettingae* (DSM 14385) and *Desulfotomaculum* sp. strain WW1

25 were isolated in our laboratory.

**Cultivation techniques.** Anaerobic culture techniques were used throughout this study. The cells were grown in an anaerobic medium typically supplemented with 0.15 g/l yeast extract. The medium contained (per liter demineralised water) 0.335 g of KCl, 4.0 g of MgCl<sub>2</sub> · 6H<sub>2</sub>O, 3.45 g of MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.25 g of NH<sub>4</sub>Cl, 10 g of NaCl, 0.10 g of

30 K<sub>2</sub>HPO<sub>4</sub>, 4.0 g of NaHCO<sub>3</sub>, 0.5 g of Na<sub>2</sub>S · 9H<sub>2</sub>O, 1.0 g of Na<sub>2</sub>SO<sub>4</sub>, 0.73 g of CaCl<sub>2</sub> · 2H<sub>2</sub>O, 10 ml of trace element and 5 ml of two-times concentrated vitamin solution which were based on medium 141 of DSM (<http://www.dsmz.de>). The medium was boiled and cooled to room temperature under a stream of O<sub>2</sub>-free N<sub>2</sub> gas. The medium



was anaerobically dispensed into serum bottles and a gas phase of 180 kPa N<sub>2</sub>/CO<sub>2</sub> (80/20, v/v) was applied. The bottles were closed with butyl rubber stoppers and sealed with crimp seals. The medium was autoclaved for 20 min at 121°C. Stock solutions of NaHCO<sub>3</sub>, Na<sub>2</sub>S, CaCl<sub>2</sub> and vitamin solutions were prepared under a nitrogen gas atmosphere and added after sterilisation. The vitamin solution contained higher amounts of vitamin-B<sub>12</sub> and vitamin-B<sub>1</sub> resulting in concentrations in the medium of 0.5 and 0.1 mg. l<sup>-1</sup>, respectively. Thiosulfate was added from 1 M filter-sterilized stock solutions. Calculated amounts of <sup>13</sup>C-methane were injected into bottles prior to autoclaving.

For coculture experiments, *A. profundus* and *Desulfotomaculum* sp. strain WW1 were grown on H<sub>2</sub> and CO<sub>2</sub> in 248 ml serum vials with 50 ml medium at 80°C and 65°C, respectively. As additional carbon source, 1 mM of sodium acetate was added. The sulphate reducers were grown for 1 day and then the gas phase was changed to 180 kPa N<sub>2</sub>/CO<sub>2</sub> (80/20, v/v) and CH<sub>4</sub> gas was added to the final concentration of 1.75 mmol per vial. The *A. profundus* culture was inoculated with *T. maritima* and the *Desulfotomaculum* sp. with *T. lettingae*. For inoculation, methane adapted cultures of *T. maritima* and *T. lettingae* were used. Since the medium already contained nearly 1 mmol of sulphate per vial, no sulphate was added.

**Analytical techniques.** Highly pure (min 99% <sup>13</sup>C) methane gas was obtained from Campro Scientific B.V. (Veenendaal, NL). Serum vials were prepared with approximately 1.75 mmol of <sup>13</sup>C-methane as substrate in 50 ml medium. Control bottles were prepared with or without <sup>13</sup>C-methane, thiosulphate and organisms. NMR-tubes contained the sample, 10% (v/v) D<sub>2</sub>O and 100 mM dioxane to give a final volume of 15 ml. The proton-decoupled <sup>13</sup>C-NMR-spectra of the samples were recorded at 75.47 MHz on a Bruker AMX-300 NMR spectrometer. For each spectrum 7200 transients (5 h) were accumulated and stored on disc using 32k data points, a 45° pulse angle and a delay time of 1 s between pulses. The measuring temperature was maintained at 10°C and the chemical shift belonging to the dioxane carbon nuclei (67.4 ppm) was used as an internal standard. The deuterium in the samples (10%, v/v) was used for field lock and dioxane as an internal standard.

Hydrogen and methane were determined at room temperature by either gas chromatography (GC) (see: Stams et al. *Appl. Environ. Microbiol.* (1993) 59 114-1119) or GC (Hewlett Packard model 5890) equipped with a mass selective detector (MS). Methane, carbon dioxide and their stable isotopes were separated on a capillary column

(innowax, 30 m x 0.25 mm (df=0.5  $\mu$ m), Packard, NL) with helium as the carrier gas. Gas samples (200  $\mu$ l) were injected in a split injector (inlet pressure 1 kPa; split ratio 25:1) at a column temperature of 35°C. Methane and carbon dioxide and their stable isotopes were monitored at  $m/z$  16 and 17, and 44 and 45, respectively. Total methane and CO<sub>2</sub> concentrations were determined quantitatively by gas chromatography (see Stams et al. (1993 *above*). The H<sup>13</sup>CO<sub>3</sub><sup>-</sup> concentration in the liquid phase was calculated from the amount of CO<sub>2</sub> which accumulated in the gas phase after acidification. Thiosulphate and sulphate were analysed by HPLC (see: Scholten and Stams, *Antonie van Leeuwenhoek* (1995) 68, 309-315). Sulphide was determined as described by Trüper and Schlegel (see: *Antonie van Leeuwenhoek* (1964) 30, 225-238).

#### **Example 1. Anaerobic methane oxidation by *Thermotoga* species**

*Thermotoga maritima* and *T. lettingae* were incubated with highly pure <sup>13</sup>C-labelled methane under strictly anaerobic conditions in the presence of thiosulphate as the electron acceptor. The experiments were carried out was studied in duplicate cultures at incubation temperatures of 80°C and 65°C, respectively. Methane oxidation and product formation were determined by analysing the gas phase by gas chromatography (GC) and GC-mass spectroscopy (MS) and by using Nuclear Magnetic Resonance spectroscopy (NMR) for liquid samples.

In acidified samples, the total CO<sub>2</sub> which accumulated in the gas phase after 40 days of incubation was measured by using GC (Table 1). In the control incubation without methane which contained totally 4.87 mmol of CO<sub>2</sub> per vial, slight growth was observed due to the presence of yeast extract in the medium and consequently, unlabelled CO<sub>2</sub> was found. Growth in the presence of <sup>13</sup>C-methane and thiosulphate by *T. maritima* and *T. lettingae* resulted in significantly increased cell numbers (Table 1). The stoichiometry of methane conversion by the two cultures yielded nearly equal amounts of products after 40 days of incubation. The percentage of <sup>13</sup>C in CO<sub>2</sub> was 5.9 % in liquid phase and 2.1 % in gas phase for *T. lettingae*. These values for *T. maritima* were 6.2% and 2.4%, respectively. The ratio of methane oxidation to carbon dioxide and thiosulphate reduction to sulphide in both samples were approximately 1:1 and 1:2, respectively. Nearly 1 mmol of <sup>13</sup>C-methane per vial was utilised by both bacteria. The rest of the methane in the vials was not utilised even after 40 days of prolonged incubations. However, when less methane was added (up to 0.5 mmol per vial), all <sup>13</sup>C-methane was completely utilised by the two bacteria. In both cases, measurable methane conversion

started after around 10 days. The rates of anaerobic methane transformation by *T. maritima* and *T. lettingae* were 32 and 30  $\mu\text{mol}$  per vial per day, respectively. The  $^{13}\text{C}$ -carbon recoveries for *T. maritima* and *T. lettingae* were calculated to be 82% and 79%, respectively.

5 Table 1 a.  $^{13}\text{C}$ -methane oxidation in the presence of thiosulphate by *T. maritima*†.

	$^{13}\text{CH}_4$	Thio-sulphate	Total $\text{CO}_2^*$	Total Sulphide #	Hydro-gen	Number of cells (ml)†
Day 0	1.75	0.99	4.87	0.05	0.00	$4 \times 10^5$
Day 40	0.68	0.00	5.75	1.96	0.05	$8 \times 10^7$
Utilised/produced	1.07	0.99	0.88	1.91	0.05	

Table 1. b.  $^{13}\text{C}$ -methane oxidation in the presence of thiosulphate by *T. lettingae*†.

	$^{13}\text{CH}_4$	Thio-sulphate	Total $\text{CO}_2^*$	Total Sulphide #	Hydro-gen	Number of cells (ml)†
Day 0	1.75	1.01	4.87	0.05	0.00	$5 \times 10^5$
Day 40	0.71	0.00	5.68	1.89	0.04	$1 \times 10^7$
Utilized/produced	1.05	1.01	0.81	1.84	0.04	

†; The values were calculated in mmol per vial and corrected with the control samples. All measurements were done in duplicates and the highest values of each sample were calculated

10 \*; Total  $\text{CO}_2$  includes  $\text{CO}_2$  from the medium composition in liquid and gas phases and  $^{13}\text{C}$ -carbon dioxide formed

#; Total sulphide includes sulphide from the medium composition in liquid and gas phases and sulphide formed during methane oxidation

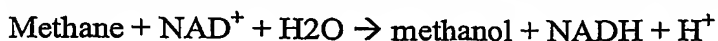
†; Ballooning cells were not taken into account during counting of the cells

#### Example 2: Biomass analysis:

15 *Thermotoga lettingae* and *Thermotoga maritima* were grown with labelled methane and thiosulfate at 65 and 80 °C, respectively. After growth, cells were centrifuged. The percentage  $^{13}\text{C}$  in the supernatant and the cell pellet were analysed. As shown before,  $^{13}\text{C}$  labelled bicarbonate was formed, but we could not detect any label incorporation in biomass. This indicates that no cell biomass is formed from methane or its degradation

products, but from yeast extract supplied to the medium. Thus, it seems that the bacteria have a split metabolism.

**Enzyme activity:** Methane-oxidizing activity was determined in cell free extracts prepared from methane grown cells. We could measure high activities of an NAD-dependent methane dehydrogenase ( $> 1 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{protein}$ ) at pH 9 and 65°C. At 80°C no activity could be measured. The apparent reaction is:



The Gibbs free energy change of this reaction is about + 90 kJ per mol. Therefore, this reaction can only occur when the concentration of the substrates are high and the concentration of the products low. We could show that the reaction is inhibited by addition of methanol to the assay mixture and that a higher activity was measured when the  $\text{NAD}^+$  concentration was increased.

### Example 3 Sulphate reduction by coculture with *Thermotoga* species

Coculture experiments were performed by growing *Archaeoglobus profundus* with *T. maritima* at 80°C and *Desulfotomaculum* sp. strain WW1 with *T. lettingae* at 65°C (Table 2). In both cases, sulphate was utilised as the electron acceptor. The ratio of  $\text{CO}_2$  formation from methane and sulphate conversion to sulphide were nearly 1:1 for both cocultures. The calculated rates of methane conversion were slower than in the case with thiosulphate, which was about 20  $\mu\text{mol}$  per vial per day. Conversion of methane coupled to sulphate reduction led to more than ten-fold increased cell numbers of both the *Thermotoga* species and the sulphate-reducing microorganisms. The  $^{13}\text{C}$ -carbon recoveries for the cocultures of *T. maritima* and *T. lettingae* were calculated to be 85% and 78%, respectively.

Table 2a.  $^{13}\text{C}$ -methane oxidation by *T. maritima* (T.m.) in coculture with *Archaeoglobus profundus* (A.p.) in the presence of sulphate.

	$^{13}\text{CH}_4$	Total Sulphate	Total $\text{CO}_2^*$	Total Sulphide #	Number of cells (ml)†
Day 0	1.75	1.10	4.87	0.11	$3 \times 10^5$ (T.m.) $2 \times 10^6$ (A.p.)
Day 40	1.07	0.31	5.53	0.85	$5 \times 10^7$ (T.m.) $7 \times 10^8$ (A.p.)
Utilised/produced	0.68	0.79	0.57	0.74	

Table 2b.  $^{13}\text{C}$ -methane oxidation by *T. lettingae* (T.L.) in coculture with *Desulfotomaculum* sp. strain WW1 (WW1) in the presence of sulphate†.

	$^{13}\text{CH}_4$	Total Sulphate	Total $\text{CO}_2^*$	Total Sulphide #	Number of cells (ml) †
Day 0	1.75	1.10	4.87	0.07	$4 \times 10^5$ (T.L.) $3 \times 10^6$ (WW1)
Day 40	1.21	0.50	5.28	0.53	$2 \times 10^7$ (T.L.) $8 \times 10^8$ (WW1)
Utilised/produced	0.54	0.60	0.41	0.51	

For symbols see Table 1.

Previous results showed that *T. maritima* and *T. lettingae* are able to grow on other  
 5  $\text{C}_1$ -compounds like  $\text{H}_2\text{-CO}_2$ , formate, methanol, and methylamine in the presence  
 thiosulphate and yeast extract<sup>18</sup>. We also observed that addition of 0.5 to 5.0 g/l of yeast  
 extract resulted in better growth and consequently slightly faster methane oxidation than  
 in the original medium. In the absence of yeast extract, the pure cultures could not grow,  
 even not on glucose. The rate of methane utilisation could be increased from 32 to 33  
 10  $\mu\text{mol}$  per vial per day for *T. maritima* and from 30 to 31  $\mu\text{mol}$  per vial for *T. lettingae*  
 when 2.5 g/l yeast extract was added in the medium. However, similarly to the findings  
 within original medium which contained 0.15 g/l of yeast extract, methane oxidation  
 started only after around 10 days of incubation. When more than 5.0 g/l of yeast extract  
 was added, growth for both organisms was better but methane oxidation rates were not  
 15 higher than the obtained values.

## Claims

1. A process for converting methane to produce hydrogen or hydrogen equivalents, *characterised* in that methane is subjected anaerobically to the activity of methane-oxidising bacteria of the order of the *Thermotogales*.
2. A process according to claim 1, wherein the methane-oxidising bacteria comprise a *Thermotoga* species.
3. A process according to claim 2, wherein the *Thermotoga* species comprises *T. maritima* or *T. lettingae*.
4. A process according to any one of claims 1-3, which is carried out at a temperature between 25 and 90°C.
5. A process according to any one of claims 1-4, which is carried out in the presence of thiosulphate.
6. A process for reducing chemical compounds by biological reduction using hydrogen equivalents, *characterised* in that the hydrogen equivalents are produced by subjecting methane to anaerobic methane-oxidising bacteria of the order of the *Thermotogales*.
7. A process according to claim 6, wherein sulphur compounds are reduced to sulphide using a sulphate-reducing species.
8. A process according to claim 7, wherein the sulphur compounds comprise sulphate and/or sulphite.
9. A process according to claim 7 or 8, wherein the anaerobic methane-oxidising species comprises a *Thermotoga*, *Thermosipho* or *Fervidobacterium* species.
10. A process according to claim 7 or 8, wherein the sulphate-reducing species comprises an *Archaeoglobus*, *Desulfotomaculum*, *Desulforomonas*, *Desulfovibrio* or *Thermodesulfovibrio* species.
11. A process according to claim 6, wherein metals are reduced from a high valence state to a low-valence or zero-valence state.

12. A process according to any one of claims 6-11, wherein a temperature of between 25 and 90°C is used.
13. A mixed culture use, containing one or more anaerobic methane-oxidising *Thermotogales* species, and one or more sulphate-reducing or metal reducing species, in particular a *Archaeoglobus*, *Desulfotomaculum*, *Desulforomonas*, *Desulfovibrio* or *Thermodesulfovibrio* species.

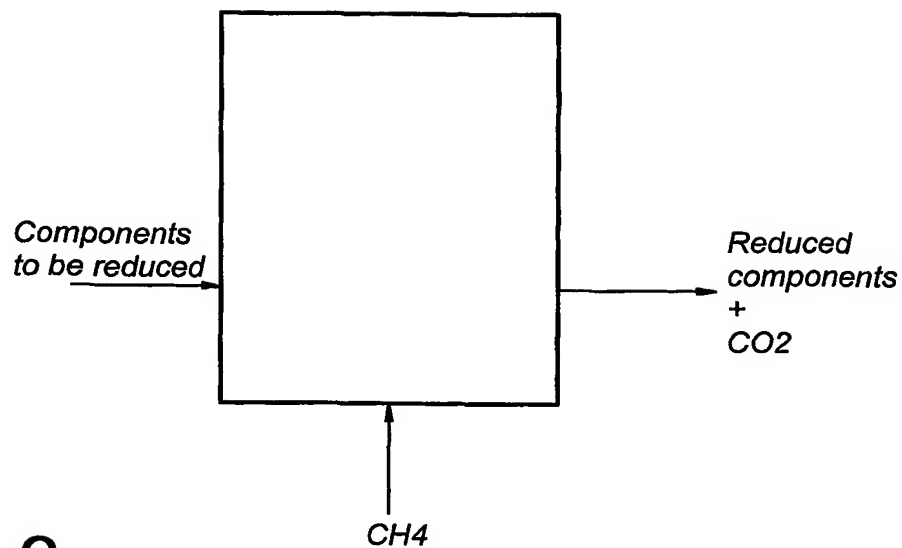
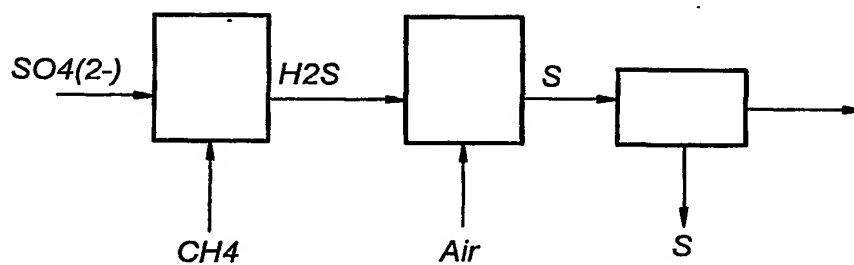
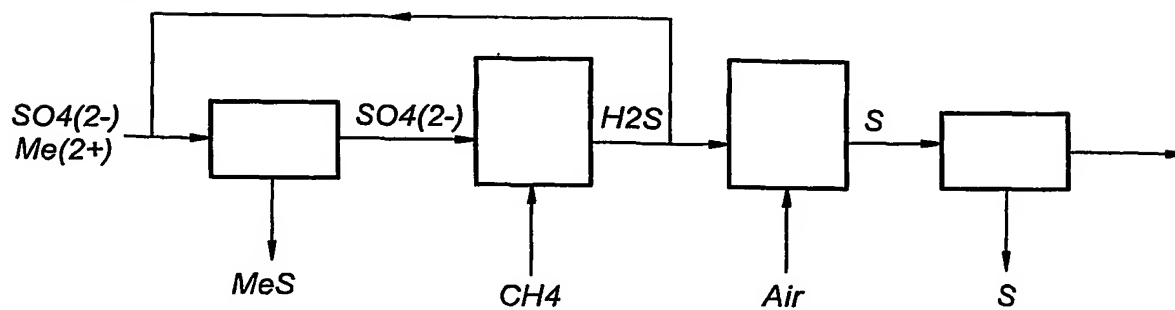
**[Received by the International Bureau on 13 April 2004 (13.04.04):  
original claim 13 amended; remaining claims unchanged; (2 pages)]**

**Claims**

1. A process for converting methane to produce hydrogen or hydrogen equivalents, *characterised* in that methane is subjected anaerobically to the activity of methane-oxidising bacteria of the order of the *Thermotogales*.
2. A process according to claim 1, wherein the methane-oxidising bacteria comprise a *Thermotoga* species.
3. A process according to claim 2, wherein the *Thermotoga* species comprises *T. maritima* or *T. lettingae*.
4. A process according to any one of claims 1-3, which is carried out at a temperature between 25 and 90°C.
5. A process according to any one of claims 1-4, which is carried out in the presence of thiosulphate.
6. A process for reducing chemical compounds by biological reduction using hydrogen equivalents, *characterised* in that the hydrogen equivalents are produced by subjecting methane to anaerobic methane-oxidising bacteria of the order of the *Thermotogales*.
7. A process according to claim 6, wherein sulphur compounds are reduced to sulphide using a sulphate-reducing species.
8. A process according to claim 7, wherein the sulphur compounds comprise sulphate and/or sulphite.
9. A process according to claim 7 or 8, wherein the anaerobic methane-oxidising species comprises a *Thermotoga*, *Thermosipho* or *Fervidobacterium* species.
10. A process according to claim 7 or 8, wherein the sulphate-reducing species comprises an *Archaeoglobus*, *Desulfotomaculum*, *Desulforomonas*, *Desulfovibrio* or *Thermodesulfovibrio* species.
11. A process according to claim 6, wherein metals are reduced from a high valence state to a low-valence or zero-valence state.



12. A process according to any one of claims 6-11, wherein a temperature of between 25 and 90°C is used.
13. A mixed culture, containing one or more anaerobic methane-oxidising *Thermotogales* species, and one or more sulphate-reducing or metal reducing *Archaeoglobus*, *Desulfotomaculum*, *Desulforomonas* or *Desulfovibrio* species.

*Fig 1**Fig 2**Fig 3*

2/2

Fig 4

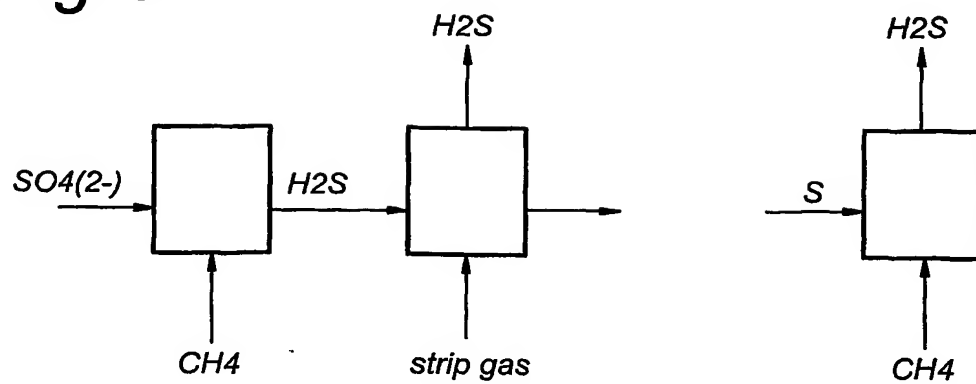
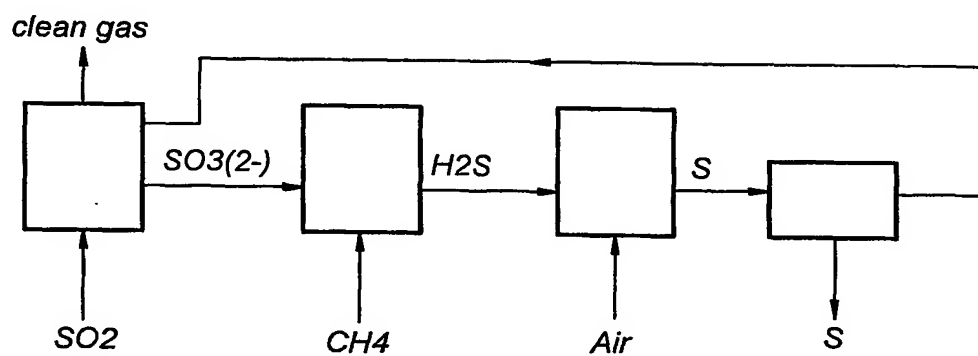


Fig 5



# INTERNATIONAL SEARCH REPORT

PCT/NL 03/00818

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 7 C12P3/00 C02F3/34		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12P C02F		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, BIOSIS, MEDLINE, WPI Data, PAJ, EMBASE		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BALK MELIKE ET AL: "Thermotoga lettingae sp. nov., a novel thermophilic, methanol-degrading bacterium isolated from a thermophilic anaerobic reactor." INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY. ENGLAND JUL 2002, vol. 52, no. Pt 4, July 2002 (2002-07), pages 1361-1368, XP008015281 ISSN: 1466-5026 cited in the application the whole document <div style="text-align: center;">--- -/-</div>	13
<div style="display: flex; justify-content: space-between;"> <span><input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.</span> <span><input checked="" type="checkbox"/> Patent family members are listed in annex.</span> </div>		
<b>* Special categories of cited documents:</b>		
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><b>*A*</b> document defining the general state of the art which is not considered to be of particular relevance</p> <p><b>*E*</b> earlier document but published on or after the international filing date</p> <p><b>*L*</b> document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p><b>*O*</b> document referring to an oral disclosure, use, exhibition or other means</p> <p><b>*P*</b> document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p><b>*T*</b> later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p><b>*X*</b> document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p><b>*Y*</b> document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p><b>*Z*</b> document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search  <div style="text-align: center;">3 March 2004</div>		Date of mailing of the international search report  <div style="text-align: center;">15/03/2004</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer  <div style="text-align: center;">Schmitz, T</div>

## INTERNATIONAL SEARCH REPORT

PCT/NL 03/00818

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>BOETIUS ANTJE ET AL: "A marine microbial consortium apparently mediating anaerobic oxidation of methane."  NATURE (LONDON),  vol. 407, no. 6804, 2000, pages 623-626,  XP002236081  ISSN: 0028-0836  abstract; figures 1,2  page 625, left-hand column, line 25 - line 28  page 625, right-hand column, line 8 - line 11  page 625, left-hand column, line 45 - line 62</p> <p style="text-align: center;">---</p>	1,6-8,13
A	<p>HOEHLER TORI M ET AL: "Field and laboratory studies of methane oxidation in an anoxic marine sediment: Evidence for a methanogen-sulfate reducer consortium."  GLOBAL BIOGEOCHEMICAL CYCLES,  vol. 8, no. 4, 1994, pages 451-463,  XP008015376  ISSN: 0886-6236  Equations 1-3  abstract  page 458, right-hand column, paragraph 2  page 459, left-hand column, paragraph 1  figures 4,6  page 461, left-hand column, line 32 - line 38  page 461, right-hand column, paragraph 4 - paragraph 5</p> <p style="text-align: center;">---</p>	1,6-8,13
A	<p>WO 02 06503 A (US ENERGY)  24 January 2002 (2002-01-24)  examples 1-18</p> <p style="text-align: center;">---</p>	
A	<p>NAUHAUS KATJA ET AL: "In vitro demonstration of anaerobic oxidation of methane coupled to sulphate reduction in sediment from a marine gas hydrate area."  ENVIRONMENTAL MICROBIOLOGY. ENGLAND MAY 2002,  vol. 4, no. 5, May 2002 (2002-05), pages 296-305, XP002236082  ISSN: 1462-2912  abstract  page 297, right-hand column, paragraph 3  page 299, right-hand column, paragraph 1;  figures 1,3-5</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	6-8,13

# INTERNATIONAL SEARCH REPORT

PCT/NL 03/00818

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>VALENTINE D L ET AL: "New perspectives on anaerobic methane oxidation."  ENVIRONMENTAL MICROBIOLOGY. ENGLAND OCT 2000,  vol. 2, no. 5, October 2000 (2000-10),  pages 477-484, XP002236083  ISSN: 1462-2912  equations 1-9  the whole document</p>	
A	<p>VALENTINE D L ET AL: "Hydrogen production by methanogens under low-hydrogen conditions."  ARCHIVES OF MICROBIOLOGY. GERMANY DEC 2000,  vol. 174, no. 6, December 2000 (2000-12),  pages 415-421, XP002236084  ISSN: 0302-8933  the whole document</p>	
A	<p>HINRICHS KAI-UWE ET AL:  "Methane-consuming archaeobacteria in marine sediments."  NATURE (LONDON),  vol. 398, no. 6730,  29 April 1999 (1999-04-29), pages 802-805,  XP002236085  ISSN: 0028-0836  abstract</p>	1,6-8,12

# INTERNATIONAL SEARCH REPORT

PCT/NL 03/00818

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 0206503	A	24-01-2002	CA	2416377 A1	24-01-2002
			EP	1301617 A2	16-04-2003
			WO	0206503 A2	24-01-2002
<hr/>					